INTERACTION BETWEEN MURINE CARDIAC STEM CELLS AND BONE MARROW-DERIVED MESENCHYMAL STEM CELLS FOR CARDIOMYOCYTE DIFFERENTIATION *IN VITRO*

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2016

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by

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

JANUARY 2016

ACKNOWLEDGEMENT

I would like to extend my utmost gratitude to my supervisor, Dr. Tan Jun Jie for his guidance with all the experiments conducted and the write-up for this thesis. His motivation and patience kept me going strong in completing my Master's Degree. Despite his busy schedule, he was always there to guide and support me throughout these years.

I would also like to thank our research officer, Puan Siti Maisura and my colleagues, Mr Ng Wai Hoe, Mr Rifqi Rafsanjani and Ms Mimi Zulaikha for their support and help throughout my time spent in Stem Cell and Heart Regeneration Research Group. I am also grateful for all the technical help I received in Advanced Medical and Dental Institute, especially from Dr. Kumitaa Theva Das and the staff of Regenerative Medicine Cluster.

No doubt, my family has been my greatest pillar of strength throughout my journey in USM. I extend my greatest thanks to my parents, my sister, my brother-in-law and my brother for always being there for me and encouraged me all these while.

Lastly, I would like to extend my special gratitude to Jabatan Perkhidmatan Awam (JPA) for offering me the Biasiswa Yang-Dipertuan Agong and supported me financially to complete my Master's Degree. I am eternally grateful and blessed with all the help and support I received, either directly or indirectly throughout these two years. Thank You.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α-SA	Alpha-Sacromeric Actin
ACE	Angiotensin Converting Enzyme
ACS	Acute Coronary Syndrome
bFGF	Basic Fibroblast Growth Factor
BMGM	Bone Marrow Mesenchymal Stem Cell Growth Medium
BM-MNCs	Bone Marrow Mononuclear Cells
BMP-2	Bone Morphogenetic Protein 2
BMP-4	Bone Morphogenetic Protein 4
CABG	Coronary Artery Bypass Grafting
CDCs	Cardiosphere-derived Cells
CdM	Conditioned Medium
cDNA	Complementary Deoxyribonucleic Acid
CGM	Cardiac Stem Cell Complete Growth Medium
CSCs	Cardiac Stem Cells
CSps	CardioStem Spheres
cTnI	Cardiac Troponin I
CVD	Cardiovascular Disease
DAPI	4',6-diamidino-2-phenylindole
Dkk-1	Dickkopf-related protein-1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco Phosphate Buffer Saline
EGF	Epidermal Growth Factor
ESCs	Embryonic Stem Cells
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
gDNA	Genomic Deoxyribonucleic Acid
HF	Heart Failure
IPSCs	Induced Pluripotent Stem Cells
LIF	Leukemic Inhibitory Factor
MI	Myocardial Infarction
MPIO	Micron-sized Particles of Iron Oxide

MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal Stem Cells
NSTEMI	Non-ST Elevation Myocardial Infarction
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium Iodide
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
SEM	Standard Error of Mean
SMA	Smooth Muscle Actinin
SP	Side Population
SRY	Sex-determining Region Y
STEMI	ST Elevation Myocardial Infarction
TGF-β1	Transforming Growth Factor-beta 1
UA	Unstable Angina
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand Factor

INTERAKSI ANTARA SEL-SEL TUNJANG JANTUNG MENCIT DAN SEL-SEL TUNJANG MESENKIMA UNTUK PROSES DIFERENSIASI SEL KARDIOMIOSIT *IN VITRO*

ABSTRAK

Latar Belakang: Sel-sel tunjang mesenkima (MSCs) terbukti dalam membantu proses pembaikan jantung melalui pengaktifan sel-sel jantung (CSCs) berikutan infaksi, tetapi pengeksploitasian secara *in vitro* melibatkan sinergi antara dua sel sebelum pemindahan sel masih kurang diketahui. ***Objektif:*** Kajian ini bertujuan untuk mengkaji kesan sinergi MSCs kepada diferensiasi sel kardiomiosit. ***Kaedah:*** CSCs yang mengekspres c-kit dan MSCs tulang diperoleh daripada mencit C57BL/6N (n=9). Diferensiasi CSCs diuji selepas diasingkan daripada MSCs berikutan ko-kultur dengan menggunakan zarah oksida besi bersaiz micron, dan dibandingkan dengan CSCs yang dirawat dengan medium terkondisi MSCs atau tanpa sebarang rawatan. Diferensiasi kardiomiosit dikesan menggunakan penwarnaan imunofluorescein dan diukur menggunakan tindakbalas polimerase berantai secara quantitatif. Semua data dianalis dengan ANOVA sehala. ***Keputusan:*** Lebih daripada 80% CSCs yang berkecambah daripada satu sel berada pada fasa G1, dengan masa penduaan populasi sebanyak 17.2 ± 0.2 jam. Populasi CSC $Sox2^{positif}$ $GATA4^{malap}$ $Nkx2.5^{malap}$ yang diinduksi dengan deksametason menunjukkan diferensiasi kardiomiosit yang lebih tinggi berbanding populasi $Sox2^{negatif}$ $GATA4^{tinggi}$ $Nkx2.5^{tinggi}$ selepas diko-kultur dengan MSCs. Hal ini terbukti dengan ekpresi yang lebih tinggi untuk cTnI, Mef2c dan GATA4 (p<0.001). Kesan ini adalah lebih besar berbanding dengan CSCs yang dirawat dengan medium terkondisi

MSCs. Akan tetapi, kesan sinergi untuk diferensiasi kardiomiosit selepas ko-kultur tidak ditunjukkan dalam populasi $Sox2^{negatif}$ $GATA4^{tinggi}$ $Nkx2.5^{tinggi}$ CSCs. Walau bagaimanapun, tiada perbezaan dalam pembentukan kardiomiosit di bawah diferensiasi yang bergantung kepada factor tumbesaran. Hal ini terbukti dengan ekpresi cTnI dan α-SA. ***Kesimpulan***: Interaksi CSCs dan MSCs secara langsung diperlukan untuk sinergi diferensiasi kardiomiosit secara *in vitro*, tetapi manfaat ini adalah terhad kepada $Sox2^{positif}$ $GATA4^{malap}$ $Nkx2.5^{malap}$ populasi. Tesis ini mentafsirkan sinergi antara MSCs dan CSCs, dan menyokong penggunaan kedua-dua sel dalam terapi klinikal.

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ABSTRACT

Background: Bone marrow-derived mesenchymal stem cells (MSCs) has been shown to facilitate heart repair via activation of endogenous cardiac stem cells (CSCs) following infarction, but little is known if the synergy of the two cells can be exploited *in vitro* prior to transplantation. ***Objective***: This study aimed to examine the synergistic effects of MSCs on CSC cardiomyocyte differentiation. ***Methods***: C-kit CSCs and MSCs were isolated from 4-6 weeks C57BL/6N mice (n=9). CSC cardiomyocyte differentiation was tested after re-isolated from MSC co-culture using micron-sized particles of iron oxide, and compared to CSCs treated with MSC-conditioned medium (CdM) or without any treatment. Cardiomyocyte differentiation was detected using immunofluorescence staining and quantified using qPCR. All data were analysed by one way ANOVA. ***Results***: More than 80% clonogenic amplified, colony-forming c-kit CSCs were at G1 phase, with a population doubling time of 17.2 ± 0.2 hr. $Sox2^{pos}$ $GATA4^{dim}$ $Nkx2.5^{dim}$ CSCs, when induced by dexamethasone, showed greater cardiomyocyte differentiation than the $Sox2^{neg}$ $GATA4^{high}$ $Nkx2.5^{high}$ CSCs after co-cultured with MSCs, as evidenced by higher cTnI, Mef2c and GATA4 expression (p<0.001). This effect was greater than CSCs treated with CdM. However, direct MSC contact did not show synergistic effects on the cardiomyocyte differentiation of $Sox2^{neg}$ $GATA4^{high}$ $Nkx2.5^{high}$ CSCs. Nevertheless, there was no difference in cardiomyocyte formation under growth

factor directed differentiation, as evidenced by the presence of cardiac troponin I and α- sacromeric actin. ***Conclusion***: Direct MSC-CSC interaction is needed to synergise CSC cardiomyocyte differentiation *in vitro*, but the benefit is confined to $Sox2^{pos}$ $GATA4^{dim}$ $Nkx2.5^{dim}$ CSCs only. This thesis defines the synergistic interactions between MSCs and CSCs, and support the use of two cells in combination for clinical therapy.

1.0 INTRODUCTION

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) remains the number one, non-communicable killer disease which recorded a mortality rate that reached 17.5 million in 2012 and accounted for 46.2% of all reported death around the globe in 2014 (Figure 1.1) [1]. In Malaysia, CVD was associated with 25.4% of all death in government hospital in 2010 [2]. This is further complicated by 43% surge in hypertension, 88% diabetes and 250% obesity in Malaysia population from year 1996 to 2006 [2], the three most common risk factors that are highly associated with acute coronary syndrome (ACS) [3]. According to Malaysian National Cardiovascular Disease Database-Acute Coronary Syndrome Registry, incidence of unstable angina (UA), non-ST elevation myocardial infarction (NSTEMI) and ST elevation myocardial infarction (STEMI) as a result of ACS accounted for 3778, 4958 and 8130 cases respectively in 2006-2010 [4]. For STEMI alone, the severe form of myocardial infarction that required immediate attention, the incidence increased by 200 cases in only 12 months [4]. With an average cost of $2000 (USD) for the treatment of one patient for an average length of stay in the hospital up to 9.2 days [5], this disease is undoubtedly imposing huge economic burden to the country.

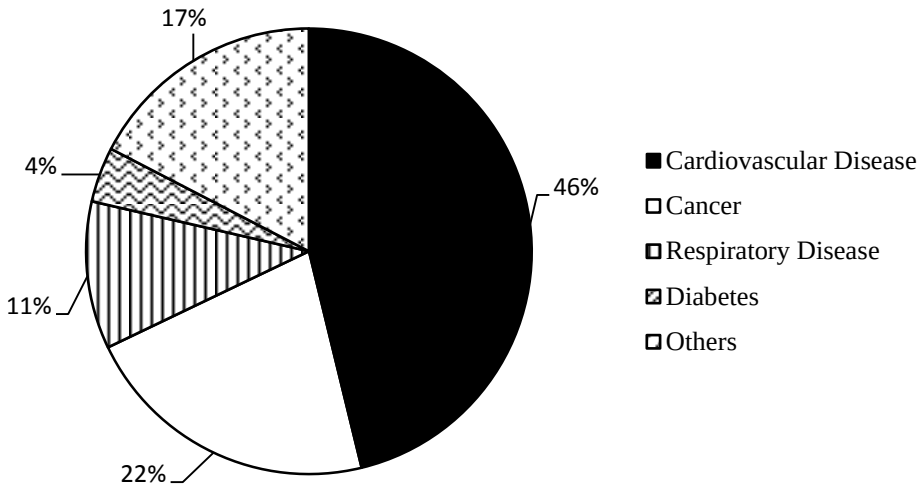

Figure 1.1: Statistics of global mortality associated with different type of non-communicable diseases in 2012 (adapted from WHO, 2014)

1.2 Myocardial Infarction and Heart Failure Pathophysiology

Myocardial infarction (MI) is a common cause of heart failure (HF). Approximately 25% of myocardial infarcted patients will develop HF, as a result of severe dysfunction of the left ventricle and progressive heart remodelling post infarction [6]. MI is due to occlusion of the main coronary artery following ruptured atherosclerotic plague and thrombosis, which diminishes the delivery of oxygen and nutrient supply to the myocardium where the vessel serves [7]. Prolonged ischemia will eventually lead to irreversible cardiomyocyte necrosis and apoptosis, followed by fibrosis and scar formation, which interrupts the contractility of the ventricular muscles [7-9]. As a compensative mechanism to maintain cardiac output in response to acute myocyte loss, the remaining cardiomyocytes undergo hypertrophy [9] as a result of increased workload [10]. If it is left untreated, the infarcted heart will eventually remodel and the alteration of the architecture will continue to weaken the cardiac contractility, and subsequently render the heart to fail.

1.3 Current Treatment for Myocardial Infarction and Heart Failure

The primary intervention for infarcted myocardium is to restore blood flow to the affected heart muscles. Patients with unstable angina and non ST elevated myocardial infarction (NSTEMI) are usually given drugs such as aspirin, clopidogrel, β-blocker, statins and angiotensin converting enzyme (ACE) inhibitor as primary management [11]. However, with the more serious ST elevated myocardial infarction (STEMI), reperfusion may be achieved with coronary artery bypass grafting (CABG) or insertion of stents in the blocked artery [3]. Although these approaches can alleviate the symptoms and improve patients' quality-of-life, the benefit is short-term as they do not replace the loss myocardium with new functional cardiomyocytes [12].

Currently, heart transplantation is the only treatment for end stage HF. However, this procedure is invasive and possesses high risk of infection and organ rejection [13-15]. Furthermore, donor heart is not readily available, making it an unfavourable option to most patients [13-15]. Recently, Ott and colleagues demonstrated a concept of producing bio-engineered heart by using the natural heart from rat [16]. The heart scaffold is de-cellularised using detergents, then re-cellularised by introducing neonatal cardiac cells and endothelial cells [16]. The bio-engineered heart started to beat after 8 days in a bioreactor that supplies electrical stimulus. However, the cardiac output was only 2% when compared to the normal adult heart [16].

1.4 Regeneration of Adult Heart using Cardiomyocyte

The rate of myocyte turnover in human was found to be very low, with annual rate of less than 1%, and the rate is inversely proportional to age [17, 18]. This explains why the heart is unable to regenerate by itself following ischemic insults. Studies showed that transplantation of neonatal cardiomyocytes engrafted and coupled with the pre-existing cardiomyocytes [19] and regenerated the heart [20, 21]. However, the source of human neonatal cardiomyocytes remains an unsolved problem to enable clinical transplantation.

1.5 Stem Cells

Stem cells are a group of primitive cells which are capable of self-renewal, proliferate and differentiate into cells with specialised function, such as cardiomyocytes [22, 23]. Stem cells are generally categorised into three groups, the embryonic stem cells, induced-pluripotent stem cells and adult stem cells.

1.5.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) are pluripotent cells originated from inner cell mass of blastocyte, with the ability to form all cells in human body except the extra-embryonic tissues [24]. The potency of ESCs make them a promising cell source to generate new cardiomyocytes, but at the same time possess the possibility of tumour formation [25, 26]. Although tumorigenesis can be prevented by using cardiac lineage committed ESCs by differentiating them *in vitro* before transplantation [27-30], only 5-20% of the total embryoid body were shown to successfully differentiated into cardiomyocytes [31]. Laflamme and colleagues rectified the low differentiation problem by creating a highly purified cardiomyocyte population by

treating ESCs with activin A and bone morphogenic protein 4 to prolong the survival and engraftment after transplantation [27]. Nonetheless, the use of human ESCs has been associated with huge ethical controversy as the procedures involve sacrifice of embryos [32-34]. In addition, the allogenicity of ESCs may subject recipient patients to long term immunosuppression in order to minimise rejection [32, 35].

1.5.2 Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are generated by reprogramming adult fibroblasts through ectopic expression of four pluripotency-associated transcription factors, namely the OCT3/4, Sox-2, c-Myc and Klf4 [36]. The generated iPSCs have similar characteristics like ESCs. As the cells can be derived from autologous skin fibroblast, the technology offers a novel source of pluripotent stem cells to generate cardiomyocytes with minimal concerns of immune rejection and ethical issues [37]. IPSCs can be differentiated into myocytes via the formation of embryoid bodies [38, 39] with exhibit spontaneous contraction when plated on gelatin-coated flask [38, 39]. Even though previous study [40] showed that iPSCs have the potential to regenerate the heart *in vitro,* the safety and feasibility of using these iPSCs *in vivo* and in early clinical trials remain a question due to possible aberrant changes in the epigenomic of the fibroblast during the reprogramming process [41].

1.5.3 Adult Stem Cells

1.5.3.1 Skeletal Myoblasts

Skeletal myoblasts are satellite cells which maintain the cellular turnover of skeletal muscles [42]. Autologous skeletal myoblasts which can be isolated from patients are multipotent and have minimal risk of tumour formation compared to pluripotent stem cells due to their restricted lineage [42]. These cells were among the first cell candidates used in clinical trials for cardiac therapy [24]. Several *in vivo* studies, as demonstrated in small and big animals such as mouse [43] , rat [44, 45], sheep [46, 47], rabbit [48, 49] and pig [50, 51] showed that these skeletal myoblasts are capable of regenerating the infarcted myocardium, by slowing the progression of heart remodelling and alleviating the left ventricular functions [52]. These data prompted the phase I clinical trials to access the safety and feasibility of using skeletal myoblasts in human [53-56]. The administration of skeletal myoblasts was found to be safe and feasible in phase I trials, and Menasche and colleagues continue with the Phase II MAGIC trials.

Table 1.1: Skeletal myoblast phase I clinical trials

Number of Patients	Changes in left ventricular ejection fraction	Reference
5	36 ± 11% - 41 ± 9% (3 months) 36 ± 11% - 45 ± 8%	[53]
10	29% - 47%	[54]
30	28% -36%	[55]
12	Control: 33.6 ± 9.3% - 38.6 ± 11% Treated: 35.5 ± 2.3 - 55.1 ± 8.2	[56]

However, the Phase II trial outcome was disappointing as restoration of left ventricular ejection fraction (p = 0.62) was not observed regardless of the number of injected skeletal myoblasts [57]. Although patients that received high dose of skeletal

myoblasts showed a significant decrease in left ventricular volume as compared to the placebo group [57], the major drawback is the high incidence of arrhythmias after skeletal myoblasts administration, with 12% of patients having arrhythmia episodes in the low dosage group and 17% in the high dosage group. The observation was attributed to the absence of gap junction connexin-43 which causes the administered skeletal myoblasts to contract independently from the pre-existing cardiomyocytes in the heart [58].

1.5.3.2 Bone Marrow-derived Stem Cells

Bone marrow mononuclear cells (BM-MNCs) are isolated via bone marrow aspiration which consist of a mixed population of regenerative and non-regenerative cells [59]. Most clinical trials using BM-MNCs as cell candidate for heart therapy showed inconsistent results (Table 1.2). Meta-analyses revealed that the benefits from BM-MNCs are modest [60-62]. Some claimed that the disappointing outcome from the trials may be a result of variation in the transplantation protocols, such as the number of administered cells and the route of administration [59]. Nevertheless, a recent study by Loffredo and colleagues re-defined the role of BM-MNCs in heart regeneration, evident by *in vivo* activation of endogenous c-kit cardiac stem cells following the administration of lin^{neg} $c\text{-}kit^{pos}$ BM-MNCs into the infarcted mouse heart [63]. In other words, BM-MNCs act through paracrine effects.

Table 1.2: List of clinical trials using bone marrow mononuclear cells

Study	Number of Patients	Dose	Effects on Ejection Fraction	References
Trials in MI				
TOPCARE-AMI (2002)	20	7.3 X 10^6	+ 16%	[64]
BOOST (2004)	60	2.4 X 10^9	+ 6.7%	[65]
BOOST# (2006)	60		Neutral*	[66]
REPAIR (2006)	204	1.98 X 10^8	+ 5%	[67]
Leuven (2006)	67	4.8 X 10^8	Neutral*	[68]
ASTAMI (2006)	97	6.8 X 10^7	Neutral*	[69]
TCT-STAMI (2006)	20	4 X 10^7	+ 10%	[70]
FINCELL (2008)	80	3.6 X 10^9	+ 7.1%	[71]
HEBE (2008)	200	2.96 X 10^8	+ 2%	[72]
ASTAMI# (2009)	97	6.8 X 10^7	Neutral*	[73]
REGENT (2009)	200	1.90 x 10^6	+ 3%	[74]
BONAMI (2010)	101	1 X 10^8	Neutral*	[75]
HEBE# (2011)	200	2.96 X 10^8	+ 4%	[76]
Late-TIME (2011)	87	1.5 X 10^8	Neutral*	[77]
TIME (2012)	120	1.5 X 10^8	Neutral*	[78]
Trials in Congestive Heart Failure				
TOPCARE-CHD (2006)	92	2.1 X 10^8	+ 2.9%	[79]
FOCUS-CCTRN (2012)	92	1 X 10^8	Neutral*	[80]

follow-up studies

* p>0.05 compared to placebo group

1.5.3.3 Bone Marrow-derived Mesenchymal Stem Cells

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) defines mesenchymal stem cells (MSCs) as cells with plastic adherence characteristic when maintained in standard culture condition, expressed CD 105, CD73 and CD 90 but not CD 34, CD 45, CD 14 or CD 11b, CD 79α or CD 19, and HLA-DR and possess the ability to form adipocytes, chondrocytes and osteoblasts *in vitro* [81]. These cells are also known to be immune-privileged due to low expression of MHC Class I and lack of MHC Class II [82, 83], which makes the cells suitable for allogeneic transplantation [83]. In contrast, Schu's group claimed that MSCs are not entirely immune-privileged [83]. MSCs treated with pro-inflammatory cytokines IFN-γ and IL-1β upregulated both MHC I and MHC II, causing around 39% of MSC lysis by T cells [83]. In addition, *in vivo* results showed that the survival of allogeneic MSCs was lower compared to syngeneic MSCs [83].

Several plausible regeneration mechanisms involving MSCs have been proposed, such as transdifferentiation into cardiomyocytes [84, 85], secretion of cardioprotective and cardiorestorative paracrine factors [86, 87] and activation and homing of cardiac stem cells to the infarct zone [88]. Transdifferentiation of MSCs into cardiac lineage can be achieved by inducing the cells with chemical such as 5-azacytidine [89-91], but the frequencies of cardiomyocytes formation from MSCs were found to be low [92]. Other studies also showed that although transdifferentiated MSC expressed cardiac specific markers, α-actinin and cardiac troponin T when co-cultured with rat or mouse myocytes *in vitro* [93, 94], the electrophysiological analysis revealed that the cells did not possess the similar electrical properties like a functional cardiomyocytes [95]. Nonetheless, several *in*

vivo studies showed MSCs improve myocardial performance even with low rate of engraftment and differentiation [96, 97], and the benefit was mainly attributed to its paracrine signalling.

MSCs are known to secrete a wide range of cardioprotective cytokines, growth factors and chemokines [98] such as IL-6, IL-8, TIMP-2, VEGF, MCP-1, SDF-1, bFGF and angiopoietin-1 [87, 99-101]. These factors have also been shown to involve in neovascularisation [102, 103] which protect further functional deterioration of the infarcted heart [104], prevent scar formation [105] and enhance proliferation, survival, recruitment and homing of cardiac stem cells to the injured site [87, 88]. More recently, exosomes that were found in MSC conditioned medium through HPLC fractionation [106, 107] have shown to exert pro-survival effects on the surviving cardiomyocytes in the infarcted heart via the activation of P13K/Akt pathway, and attenuate heart remodelling and restore cardiac functions post administration [107].

Three high profile clinical trials, the POSEIDON [108], the PROMETHEUS [109] and the MSC-HF [110] trials were initiated to test the safety, feasibility and efficacy of MSC therapy in injured myocardium. In POSEIDON, both allogeneic and autologous BM-MSCs in treating patients with ischemic cardiomyopathy were found safe with no observed adverse effects following the therapy [108]. PROMETHEUS and MSC-HF administered autologous BM-MSCs to patients underwent coronary artery bypass grafting [109] and chronic ischemic heart failure [110], respectively. All these trials showed promising improvement in overall global functions after MSC administration.

1.6 Cardiac-derived Stem Cells

The heart was once thought to be a terminally differentiated organ and the paradigm was used for decades until it was challenged by the discovery of cardiac-derived stem cells (CSCs) [22]. The endogenous CSCs were found to be multipotent, self-renewing and capable of forming cardiomyocytes, smooth muscle cells and endothelial cells [22, 23]. These primitive cells are thought to be responsible for cardiac cellular homeostasis in the heart [111]. Several types of CSCs have been identified and isolated based on their surface marker and *in vitro* characteristics, namely the c-kit [22, 112], Sca-1 [113, 114], Isl-1 [115, 116], cardiac side population [117, 118], cardiospheres [23] and cardiosphere-derived cells [119, 120].

1.6.1 C-kit^{pos} Cardiac Stem Cells

The first reported primitive CSCs present in the heart were isolated based on the expression of stem cell factor receptor CD 117, or c-kit. These cells do not express CD 34 and CD45 [22, 23, 121]. C-kit^{pos} CSCs were also shown to play an important role in cardiomyogenesis in embryonic and neonatal heart development [122]. In adult heart, most of these cells were found to reside in the atrium and the ventricular apex, albeit at a very low density (1 cell in every 10,000 myocytes) [22]. Owing to the scarcity of the cells, optimised protocol has been developed to isolate and expand these cells, which can be maintained up to 40 passages *in vitro* without affecting the stemness and characteristics [123].

Pre-clinical studies showed that these c-kit^{pos} CSCs can regenerate both the rat [22, 112, 124] and mice [23, 125] hearts post infarction via the formation of new myocytes and vasculatures, and protect the pre-existing cardiomyocytes from

apoptosis through IGF-1 secretion [126, 127]. An elegant experiment by Ellison and colleagues revealed the significant role of c-kit CSCs in endogenous heart repair through total ablation of proliferating cells in the heart by 5-flurouracil administration into isoproterenol-induced infarcted mice [112]. The absence of proliferative cells blunted the recovery of the injured heart and the effect could then be reversed through administration of c-kit^{pos} CSCs, evident by new myocyte formation [112].

Phase I clinical trial, the Stem Cell Infusion in Patients with Ischemic cardiomyopathy trial, or SCIPIO, was initiated to treat patients with ischemic cardiomyopathy with c-kit^{pos} CSCs [128, 129]. The trial showed that c-kit^{pos} CSCs (1 million) injection increased left ventricular ejection fraction and decreased scar tissues after 4 and 12 months of administration, with no reported adverse effects [128, 129]. The safety concern in regard to c-kit^{pos} CSC megadose was addressed by administering 20 million cells into swine heart [130], which showed neither detrimental effects on renal and liver functions, nor resulted in myocardial injury or impairment of left ventricle function [130].

1.6.2 Sca-1^{pos} Cardiac Stem Cells

Stem cell antigen-1 (Sca-1) belongs to the lymphocyte activation protein-6 (Ly6) gene family and was previously used in isolating hematopoietic stem cells [131]. Sca-1^{pos} CSCs were first identified in adult mouse heart by Oh and colleagues [113] and expressed the cardiac transcription factors, GATA-4, Mef2c and TEF-1 but not the hematopoietic markers (CD 45, CD 34, c-kit, Lmo2, GATA-2, and Tal/Scl2 protein) and the endothelial markers (CD34, Flk1 and Flt-1) [113]. In human, the Sca-1-like CSCs was found mostly in the atrium, including the intra-atrium septal as

well as the atrium-ventricular boundary [114]. *In vitro* studies showed that these Sca-1 expressing CSCs were capable of differentiating into beating clusters with 5-azacytydine [113] or oxytocin [132] treatment and expressed both the cardiac transcription factors, GATA-4 and Nkx 2.5 [114, 132, 133] and the structural proteins such as α-sacromeric actin, cardiac troponin I, cardiac troponin T and myosin heavy chain [113, 132]. Transplantation of Sca-1^{pos} CSCs was shown to attenuate heart remodelling *in vivo* [134]. In addition to the observed increase in left ventricular ejection fraction post administration, Sca-1^{pos} CSC also induced vascularization in the peri-infarct zone [134]. The regenerative function of Sca-1^{pos} CSCs is likely due to the secretion of SDF-1, which was shown to provide cardioprotective effects by preserving the infarcted heart and promote cell surviving through the STAT3 signalling pathway [135, 136].

1.6.3 Isl-1^{pos} Cardiac Progenitor Cells

The LIM-homeodomain transcription factors Isl-1 expressing cardiac progenitor cells, or cardioblasts were first described by Laugwitz and colleagues in rat, mouse and human heart [115]. Isl-1^{pos} CSCs were found abundantly in neonatal heart and are involved in cardiogenesis [137]. These cells express GATA-4 and Nkx2.5, but not Sca-1 or c-kit [115, 138] and have shown to developmentally contribute to the growth of the secondary heart field which include the right ventricles and the outflow tract [115, 138]. The embryonic stem cell-derived Isl-1^{pos} progenitor cells are capable of forming cardiomyocytes, smooth muscle cells and vascular endothelial cells and the mechanisms are largely controlled by the Wnt/β-catenin pathway [116, 139]. However, the number of these Isl-1^{pos} cells in the heart after birth is extremely low, with only 500-600 cells detected in 1-5 day old postnatal rat [115].

1.6.4 Cardiac Side Population Cells

Cardiac side population (SP) cells were identified based on its ability to efflux DNA binding dye Hoechst 33342 by the ATP-binding cassette transporter (ABCG2) [117, 118]. These transporters enable the cells to excrete cytotoxic products, protect them from apoptosis [140], enhance its proliferation and inhibit myogenic differentiation *in vitro* [141]. Approximately 0.03% to 3.5% of SP cells can be isolated from adult heart using FACS sorting [117, 142-144]. When co-cultured with cardiomyocytes, more than 30% of these SP cells were shown to differentiate into mature cardiomyocytes [143, 145]. In addition, these SP cells were also able to differentiate into endothelial lineage when treated with vascular endothelial growth factor (VEGF) for 28 days [146].

1.6.5 Cardiospheres and Cardiosphere-derived Cells

Cardiospheres are 20-150 μm spheres generated from explant outgrowth from heart biopsies [23, 119]. These cardiospheres consist of stem/progenitor cells which reside in the core and cardiac lineage committed cells and differentiated cells which comprise the outer layer of the spheres [23]. The three dimensional microenvironment of cardiospheres was shown to protect the c-kit^{pos} CSCs from oxidative stress as well as maintain its stemness and functions [147]. When these cardiospheres were expanded on fibronectin, the cardiosphere-derived cells (CDCs) become highly proliferative in monolayer, and are multipotent and clonogenic [148]. This enables fast expansion of the cells for heart therapy, with retained regenerative potentials [119, 120, 149]. The CDCs are heterogeneous and expressed stem cell markers; c-kit, Oct 3/4, Sox-2 and Klf4 as well as the mesenchymal markers; CD 90 and CD 105 [120]. The therapeutic effects of CDCs have also been demonstrated in

many *in vivo* studies, ranging from small to big animals [149-151] and in human trials [152, 153]. These CDCs showed potentials in reducing infarct size, improving left ventricular ejection fraction and cardiac hemodynamic in infarcted animal models [149, 150] and the benefits could be maintained up to 16 weeks [120]. The positive observation in *in vivo* studies led to the initiation of randomised phase I clinical trial, the CArdiosphere-Derived aUtologous stem Cells to rEverse ventricUlar dySfunction study, or the CADUCEUS trial conducted in the United States [152]. However, no difference in left ventricular ejection fraction and end systolic/diastolic volume were observed with CDC administration despite significant reduction in scar mass [152].

1.7 Cell Therapy with Combination of Different Stem Cells in Single Administration

Most of the *in vivo* pre-clinical testing and clinical trials employ only single type of stem cells to examine its therapeutic efficacy in regenerating damaged myocardium. Regardless of the types, the density of CSCs in the adult heart is generally low [22, 115, 117]. Thus, extensive expansion is required to scale up the cell number for therapy, which can also be time consuming. To reduce the time period for cell expansion, combining different types of stem cells may serve as an alternate solution. The idea of combining two different types of stem cells stemmed from a report by Winter and colleagues, who showed better cardiac performance with greater amelioration of ejection fraction deterioration in animals when epicardium-derived cells were co-cultured and co-transplanted with Sca-1 cardiac progenitor cells [154]. Similarly, combination of cardiac stem cells and circulatory angiogenic cells also showed superior global improvement compared to subjects that received single cells [155]. This concept prompted an *in vivo* study by Williams and colleagues to use

candidates which have been brought to clinical testing, the human c-kit^{pos} cardiac stem cells and bone marrow mesenchymal stem cells [156]. The study showed that the regenerative effects following co-administration of both MSCs and CSCs were greater compared to single cell-treated groups [156]. This excitement prompted series of experiments described in this thesis to decipher the synergy between mesenchymal stem cells and the c-kit expressing CSCs and exploit the interactions during CSC expansion *in vitro.*

1.8 Magnetic Re-Isolation of Iron-Labelled Cells from Mixed Cell Culture: The Novel Use of Micron-Sized Iron Oxide Particle

Micron-sized iron oxide particles (MPIO) are fluorescent microspheres made of polystyrene-divinyl benzene polymer with a magnetic core [157] which were widely used as a contrast agent for *in vivo* cellular tracking using magnetic resonance imaging (MRI) [120, 158, 159]. Due to its non-toxic, biologically inert characteristics, MPIO can easily be incorporated into multiple cell lines and retained in the labelled cells for at least 6 weeks post-transplantation, though with some degree of dilution due to mitosis [160]. Furthermore, the iron-labelled cells can also be magnetically recruited to the injured myocardium *in vivo* [161-163]. This suggests that iron-labelled cells can be mobilised to a target site with the aid of a magnet without affecting cell viability. Hence, this study also sought to apply MPIO for re-isolating iron-labelled cells from a mixed cell population in *in vitro* culture.

PROBLEM STATEMENT AND OBJECTIVES

Problem Statement:

Heart contains endogenously-derived, c-kit expressing cardiac stem cells (CSCs) with regenerative capability. Mesenchymal stem cells (MSCs) had been shown to facilitate endogenous heart repair, but little is known about the synergy between endogenous cardiac stem cells and bone marrow-mesenchymal stem cells which can potentially be exploited during *in vitro* expansion prior to transplantation.

Main objective

To determine synergistic effects between cardiac stem cell and mesenchymal stem cells on CSC cardiomyocyte differentiation *in vitro*.

The specific objectives of this study were:

1. To isolate and characterise adult mouse cardiac stem cells and bone marrow derived mesenchymal stem cells from C57BL/6N mice.
2. To determine whether cardiac stem cells can be re-isolated from mesenchymal stem cell co-culture by using micron-sized iron oxide particles.
3. To identify the mode of synergistic interaction between cardiac stem cells and mesenchymal stem cells to exert superior cardiomyocyte differentiation *in vitro*.

2.0 METHODOLOGY

2.1 Endogenous Cardiac Stem Cell Isolation, Culture and Expansion

The protocol for CSC isolation was adapted from Smits *et al.* [133] with slight modifications. All procedures were conformed to USM animal ethical approval (USM/Animal Ethics Approval/ 2011/ (74) (357)). Briefly, CSCs were isolated from 4-6 week-old C57BL/6N mice hearts (n=9). All mice were euthanised by carbon dioxide inhalation. The isolated hearts were removed and kept in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco®, Invitrogen Life Technologies Co., Carlsbad, CA, USA) supplemented with 10% Foetal Bovine Serum (Gibco®, Invitrogen Life Technologies Co., Carlsbad, CA, USA) and 1x Penicillin and Streptomycin (Gibco®, Invitrogen Life Technologies Co., Carlsbad, CA, USA). The hearts were washed twice with cold M-buffer (see appendix II) to remove blood cells. The isolated hearts were then transferred to a 100 mm cell culture-treated petri dish and minced into pieces with ~1mm^3 in size, after which was followed by two washes with cold Dulbecco phosphate buffer saline (DPBS) (Gibco®, Invitrogen Life Technologies Co., Carlsbad, CA, USA). The supernatant was removed and the heart tissues were allowed to settle at the bottom of conical tube before being treated with 1 mg/ml collagenase A (Roche Applied Science, US) for 2 hr at 37˚C in a waterbath. The tissues were vigorously shaken from time to time to facilitate digestion. After that, the digested heart tissues were filtered into a 50 ml conical tube through a 40 μm cell strainer (BD Biosciences Franklin Lakes, NJ, USA), followed by 5 washes with cold M-buffer (5 ml each) to separate cardiomyocytes and cardiac small cells. Then, the filtered cell suspension was centrifuged at 300 *g* for 5 min at room temperature and the procedure was repeated

once by re-suspending the cell pellet with 5 ml of cold M-buffer. After that, the cells were re-suspended in incubation medium (see appendix II) and counted using a haemocytometer prior to sorting with using EasySep® Mouse CD117 (c-kit) selection cocktail (STEMCELL Technologies, Vancouver, Canada) according to manufacturer's protocols. Briefly, about 2 x 10^6 of the isolated cells were re-suspended in 500 µl of incubation medium in a 5 ml polystyrene round bottom tube. Then, phycoerythrin (PE)-tagged CD 117 (c-kit) antibody (25 µl) was added and the cells were incubated for 15 min at room temperature. Then, EasySep® PE selection cocktail (STEMCELL Technologies, Vancouver, Canada) comprising monoclonal bispecific tetrametric antibody complex which targets PE and dextrose (35 µl), were added and incubated for 15 min at room temperature. This was then followed by incubation with magnetic dextran nanoparticles (25 µl) at room temperature for 10 min. The mixture was topped up with incubation medium to 2.5 ml and the tube was put into a EasySep® magnet (STEMCELL Technologies, Vancouver, Canada) for 5 min. Supernatant was discarded by inverting the tube and the magnet, and the labelled cells that retained in the tube were re-suspended in the incubation medium. The procedure was repeated up to four times. Positively selected c-kit^{pos} CSCs were re-suspended in fresh cardiac stem cell complete growth medium (CGM, see appendix II). The isolated CSCs were cultured in culture flasks coated with 1.5% (w/v) gelatin (Sigma-Aldrich, St Louis, MO, USA), at a density of ± 10,000/cm^2 with CGM. For routine passaging, confluent CSCs were washed twice with pre-warmed DPBS, detached using 0.05% Trypsin-EDTA (Gibco®, Invitrogen Life Technologies Co., Carlsbad, CA, USA) at 37˚C and centrifuged at 330 *g* for 3 min. Supernatant was discarded and the cell pellet was re-suspended in fresh CGM.

2.2 CSC Colony Forming Unit and Cloning Assay

As c-kit expressing CSCs were heterogeneous, this study employed two selection steps to obtain clonogenic, homogeneous population after magnetic sorting. First, the isolated CSCs were expanded up to three passages, prior to plating at ± 120 cells/cm^2 in CGM, and allowed to grow for 14 days in a 100 mm petri dish pre-coated with 1.5% gelatin. Cell colonies with at least 20 cells and 1-8 mm in diameter were selected for subsequent clonogenic expansion. The cell colonies were selectively isolated by trypsinisation within a hydrophobic barrier drawn around the colony using ImmEdge hydrophobic barrier pen (Vector Laboratories, CA, USA). Secondly, the isolated colony forming cells were then seeded into a gelatin-coated 96-well plate at a density of one cell in every two wells. The plates were incubated for an hour inside a 5% CO_2 incubator at 37˚C before checking under the microscope. Wells that had only one cell were counted while wells with ≥ 2 or no cells will be excluded. CSCs were allowed to expand from single cells under standard culture conditions for 2 weeks. Cloning efficiency was calculated using the formula as below.

$$\text{Cloning efficiency} = \frac{\text{Number of clones generated from single cell}}{\text{Number of well with single cell at day 0}} \text{ X } 100\%$$

2.3 Murine Bone Marrow-derived Mesenchymal Stem Cell Isolation, Culture and Maintenance

Bone marrow-derived mesenchymal stem cells (MSCs) were isolated from the femur and tibia bones of 4-6 weeks old C57BL/6N mice. Both the epiphysis end of the bones were cut open with sterile scissors and flushed through using 27-29G needles with incubation medium into a 50 ml conical tube until the bones became clear. The cells were centrifuged at 380 *g* for 5 min and re-suspended in fresh bone marrow mesenchymal stem cell growth medium (BMGM, see appendix II). Bone marrow cells were expanded up to passage 2 prior to sorting. All lineage-committed cells within the isolated MSCs were depleted using EasySep® Mouse Hematopoietic Progenitor Cell Enrichment Kit (STEMCELL Technologies, Vancouver, Canada) according to manufacturer's protocols. Briefly, the bone marrow cells were re-suspended in 500 µL incubation medium in a 5 ml polystyrene tube. Then, rat serum (25 µl) was added to the cells followed by 25 µl of EasySep® mouse hematopoietic progenitor cell enrichment cocktail. The cells were incubated on ice for 15 min. After that, EasySep® biotin selection cocktail (50 µl) was added and the cells were incubated on ice for 10 min. This was followed by incubation with 25 µl of magnetic nanoparticles on ice for 10 min. The solution was then brought up to 2.5 ml with incubation medium and left in the EasySep® magnet (STEMCELL Technologies, Vancouver, Canada) for 3 min. All labelled committed cells were retained in the magnet and lineage depleted MSCs were isolated from the supernatant. The cell was then centrifuged at 380 *g* for 5 min and maintained in fresh BMGM. Passage 10-11 MSCs were used for all experiments described in this thesis.

2.4 Flow Cytometry

To minimise the destruction of surface markers, cells were detached using TryPLE Express (Gibco®, Invitrogen Life Technologies Co., Carlsbad, CA, USA) and centrifuged at 380 *g* for 5 min. Then, the cells were re-suspended in incubation medium and cell number was determined using a haemocytometer. Approximately 2 x 10^5 cells were added into one 5 ml polystyrene tube. Cells were labelled with fluorescein isothiocyanate (FITC) and PE conjugated antibodies with dilutions as described in Table 2.1 at 4˚C in dark for 1 hour. The labelled cells were washed thrice with 500 µL DPBS at 300 *g* for 5 min prior to analysis with BD FACSCanto II flow cytometer (BD Biosciences Franklin Lakes, NJ, USA).

Table 2.1: List of primary and secondary antibodies and its dilution factor for MSC characterisation

Marker	Catalogue Number	Antibody Dilution	Manufacturer
CD 29-FITC Clone HMβ1-1	130-102-503	1:10	Mitenyi Biotech
CD 44-FITC Clone IM 7.8.1	130-102-511	1:10	Mitenyi Biotech
CD 105-PE Clone MJ7/18	130-102-548	1:10	Mitenyi Biotech
Sca-1-FITC Clone D7	557405	1:50	BD
CD 34-FITC Clone RAM 34	560238	1:50	BD
CD 45-FITC Clone 30F11	130-102-491	1:10	Mitenyi Biotech
CD 90.1-FITC Clone His51	130-102-635	1:10	Mitenyi Biotech
CD 90.2-FITC Clone 53-2.1	553003	1:50	BD

2.5 Immunofluorescence Labelling

Cells were re-suspended at a density of 1 x 10^5 cells/ml and cytocentrifuged onto a glass slide using Cytospin™ 4 Cytocentrifuge (Thermo Scientific, Logan, UT, USA). Then, the cells were fixed with Shandon Cell-Fixx spray fixative (Thermo Scientific, Logan, UT, USA) and kept at room temperature. Prior to staining, the samples were treated with 95% ethanol to remove the wax for 15 min. For staining of intracellular proteins, samples were permeabilised using 0.1% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) for 10 min. Then, the samples were washed thrice with 0.1% Tween-20 (Sigma-Aldrich, St Louis, MO, USA) before blocking for 30 min in a humidifier chamber using blocking buffer with 10% donkey serum (Millipore, Billerica, MA, USA) to prevent unspecific binding. Samples were incubated with primary antibody overnight at 4˚C after diluted with 0.1% Tween-20 at specific ratio (Table 2.2). The samples were washed thrice with 0.1% PBS-Tween, followed by counter-labelling with secondary antibody diluted at 1:1000 in 0.1% PBS-Tween-20 at 37˚C for 1 hr in the dark. After 3 washes with 0.1% PBS-Tween-20, the samples were counterstained with nuclei staining, 1 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) for 14 min at room temperature. The slides were then mounted with VectaShield Mounting Medium (Vector Laboratories, CA, USA) with coverslips and sealed with clear nail polish around the edges for long term preservation. The samples were imaged with IX41 fluorescence microscope (Olympus, Japan) and the contrast was adjusted using Image J. The similar procedure applied to staining cells which were grown on Nunc Lab-Tek 8 well chamber slide (Thermo Scientific, Logan, UT, USA) after fixing with 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) on ice for 20 min.

Table 2.2: List of antibodies used in this study and its dilution factor

Primary Antibody	Manufacturer & Dilution (catalogue number)	Secondary Antibody (Alexa Fluor® 488)
CD 117 (H-300) Rabbit Polyclonal IgG	Santa Cruz 1:50 (sc-5535)	donkey anti-rabbit 1:1000
Nkx 2.5 (H-114) Rabbit Polyclonal IgG	Santa Cruz 1:50 (sc-14033)	donkey anti-rabbit 1:1000
GATA-4 (H-112) Rabbit Polyclonal IgG	Santa Cruz 1:50 (SC-9053)	donkey anti-rabbit 1:1000
α-sacromeric actin Clone EA-53 Rabbit anti-mouse	Sigma-Aldrich 1:200 (A 7811)	donkey anti-rabbit 1:1000
Smooth muscle actinin Clone 1A4 Mouse anti-mouse	Sigma-Aldrich 1:500 (A 2547)	donkey anti-mouse 1:1000
Von Willebrand factor Rabbit anti-human	Dako 1:200 (A 0082)	donkey anti rabbit 1:1000
Cardiac Troponin I (H-170) Rabbit anti-mouse	Santa Cruz 1:50 (sc-15368)	donkey anti-rabbit 1:1000
Myosin Heavy Chain Clone 3-48 Mouse Monoclonal IgG	Abcam 1:200 (AB 15)	donkey anti-mouse 1:1000
Anti-human nuclei Clone 235-1 Mouse anti-human	Millipore 1:50 (MAB 1281)	donkey anti-mouse 1:1000

2.6 Cell Cycle Assay

Cell samples (1 x 10^6) were added and incubated with PI staining solution consisted of 250 μg/ml propidium iodide (PI) (Gibco®, Invitrogen Life Technologies Co., Carlsbad, CA, USA), 800 μg/ml RNase (Gibco®, Invitrogen Life Technologies Co., Carlsbad, CA, USA), and 0.8% Triton X-100 in DPBS in a 5 ml polystyrene tube. The cells were gently vortexed and incubated for 10 min at room temperature in the dark before they were examined using flow cytometer (BD FACSCanto II). All data were analysed using ModFitLT™ software. DNA QC particles (BD Biosciences Franklin Lakes, NJ, USA) consisted of chicken erythrocyte nuclei and calf thymocyte nuclei were used as the controls.